DESCRIPTION

UTILIZATION OF CELL FORCIBLY EXPRESSING TOLL-LIKE RECEPTOR

Technical Field

The present invention relates to use of cells forced to express a Toll-like receptor.

Background Art

Two immune systems, innate and acquired, are known in a living body. In contrast to acquired immunity, which is found only in higher organisms such humans, the innate immune system is conserved from insects to humans in a wide range of organisms. When external pathogenic bacteria or such invade, cells responsible for the innate immunity (for example, macrophages and dendritic cells) function as the first-stage prevention of infections: quickly detect pathogenic bacteria or such, directly attack the pathogenic bacteria or such through phagocytosis or such, and release alarm signals such as cytokines to activate the acquired immune system. Toll-like receptors (TLRs) play a role in the first detection of bacteria in a series of immune responses. Ten types of TLRs have been identified in human so far, each being considered a receptor recognizing a different molecular structure (Non-Patent Document 1). Of these, TLR9, which was first reported by the research group of Akira *et al.* in 2000, was identified as a receptor that recognizes bacterial DNA, in particular the CpG motif (Non-Patent Document 2 and Patent Document 1).

Previous studies have reported the effect of lipopeptides on the expression activity of NF-κB in CHO cells that were forced to express human TLR2, as well as the effect of various CpG DNA motifs derived from pathogenic E. coli on cytokine (IL-8) yield in HEK293 cells that were forced to express human TLR9 (Non-Patent Documents 3 to 5).

Information on prior art documents relevant to the invention of the present application is listed below.

[Patent Document 1] Japanese Patent Application Kokai Publication No. (JP-A) 2002-34565 (unexamined, published Japanese patent application)

[Non-Patent Document 1] O. Takeuchi, S. Akira, International Immunopharmacology 1 (2001) 625-635

[Non-Patent Document 2] H. Hemmi *et al.*, Nature 408 (2000) 740-745

[Non-Patent Document 3] Yoshimura A., Takada H., Kaneko T., Kato I., Golenbock D., Hara Y., "Structural requirements of muramylpeptides for induction of Toll-like receptor 2-mediated NF-kappaB activation in CHO cells." Journal of Endotoxin Research 6 (5): 407-20, 2000.

[Non-Patent Document 4] Klinman DM., Takeshita F., Gursel I., Leifer C., Ishii KJ., Verthelyi D.,

Gursel M., "CpG DNA: recognition by and activation of monocytes." Microbes & Infection 4 (9): 897-901, 2002 Jul.

[Non-Patent Document 5] Takeshita F., Leifer CA., Gursel I., Ishii KJ., Takeshita S., Gursel M., Klinman DM., "Cutting edge: Role of Toll-like receptor 9 in CpG DNA-induced activation of human cells." Journal of Immunology 167 (7): 3555-8, 2001 Oct 1.

Disclosure of the Invention

The present inventors have revealed that certain DNA motifs derived from dairy lactic acid bacteria, including probiotic lactobacilli, can exhibit immuno potentiation of the intestinal tract immune system (H. Kitazawa *et al.*, Int. J. Food Microbiol. 65 (2001) 149-162; H. Kitazawa *et al.*, Int. J. Food Microbiol. (2003) in press). This finding implies that TLR9 recognizes not only DNA from pathogenic bacteria, but also DNA from dietary lactobacilli, and thus contributes to immunity activation. In the future, TLR9 can become an important tool not only in the negative evaluation of pathogenic bacteria but also in the development of functional food products using dairy lactic acid bacteria (LAB), provided that recognition towards various DNA motifs and systems for activity assessment can be established.

In the development of functional food products, it is necessary to evaluate their ultimate effects on human, and for obtaining basic findings, investigation using experimental animals and animal cells is essential. To this end, the present invention focused on pigs as an experimental animal, which has great potential utility as a human model system from aspects of organ transplantation and such, and is of great significance in food industry. In order to establish cells in which TLR9 is forcibly expressed for use in systems of assessing functional DNA, the present inventors decided to clone a swine TLR9 gene, and introduce the gene to forcibly express it in animal cells.

The present invention has been made in view of the above conditions. An objective of the present invention is to provide uses of cells with forced TLR9 expression.

The present inventors cloned from the Peyer's patches of a swine intestinal tract a gene of Toll-like receptor 9, which is a receptor protein that recognizes the CpG DNA motif derived from pathogenic bacteria, and established animal cells (transfectants) in which swine TLR9 (sTLR9) is forcibly expressed. The presence of the sTLR9 protein in these animal cells is confirmed by generation and use of polyclonal antibodies against sTLR9. The sTLR9 transfectant was analyzed for its functionality on CpG DNA, and its application to systems for assessing LAB's DNA activity was sought.

Specifically, this was carried out according to the following (1) through (5):

(1) Total RNA was extracted from the Peyer's patch of a swine intestinal tract. Using primers prepared from highly conserved regions of human and mouse TLR9 genes, RT-PCR and RACE

were performed to clone the swine TLR9 gene. The gene's full-length sequence was determined.

(2) The full-length amino acid sequence of swine TLR9 obtained from the genetic information was screened for antigenic determinant sites. The selected region was synthesized by peptide synthesis and used as an antigen for generating a swine TLR9 polyclonal antibody. Rabbits were immunized with the chemically synthesized antigen to generate polyclonal antibodies against swine TLR9 using standard techniques.

(3) HEK293T cells (human embryonic kidney cells) were transfected with the swine TLR9 gene to establish swine TLR9 gene-transfected cells (transfectant).

(4) SwineTLR9 expression in the HEK293T cells was confirmed by detecting swine TLR9 mRNA expression using RT-PCR. The expression of the swine TLR9 membrane protein was confirmed by immunostaining with a swine TLR9 polyclonal antibody using laser microscopy and flow cytometry.

(5) The reactivity of swine TLR9 against oligodeoxynucleotides (CpG2006 and CpG1826), which contain specific CpG DNA motifs that strongly stimulate human and mouse cells respectively, was analyzed.

The swine TLR9 gene, as revealed from the analysis result, is consisted of 3090 bases encoding 1029 amino acid residues (MW: 115.8). A 3145bp-long cDNA sequence comprising the swine TLR9 gene was determined. The amino acid sequence of swine TLR9 shows an extremely high homology to human TLR9 (82.9%) and a 74.9% homology to mouse TLR9, therefore swine TLR9 shows a relatively higher homology to human TLR9 than to mouse TLR9. The results of RT-PCR and immunostaining with a swine TLR9 polyclonal antibody revealed that the swine TLR9 protein was expressed as a membrane protein in the swine TLR9 transfectant, indicating successful creation of the swine TLR transfectant. Functional analysis conducted against CpG DNAs using this transfectant indicated that swine TLR9 has a higher reactivity with CpG2006 than with CpG1826. This analysis revealed that swine TLR9 can recognize a human-specific CpG DNA motif more effectively than a mouse-specific CpG DNA motif. Surprisingly, the results of comparing the levels of mRNA expression in various tissues by real-time PCR revealed that the mRNA expression in the Peyer's patches and mesenteric lymph nodes, which are tissues that have a central role in the intestinal tract immune system, was three or more times higher than in that of the spleen.

Accordingly, the present invention provides the following (1) to (21):

[1] A method for assessing whether a test sample activates the intestinal tract immune system, comprising the steps of:

(a) contacting a test sample with a cell forced to express an intestinal tract tissue-expressed Toll-like receptor; and

(b) measuring activity of the Toll-like receptor using signal transduction in the cell as an indicator,

wherein the test sample is judged to be activating the intestinal tract immune system if the activity of the Toll-like receptor is increased as compared to activity of the Toll-like receptor in a cell not contacted with the test sample;

[2] a method of screening for a sample that activates the intestinal tract immune system, comprising the steps of:

(a) assessing whether a plurality of test samples activate the intestinal tract immune system by the assessment method of [1]; and

(b) selecting from the plurality of test samples those assessed to activate the intestinal tract immune system;

[3] a method for producing a pharmaceutical composition that activates the intestinal tract immune system, comprising the steps of [2] and a further step of mixing the sample assessed to activate the intestinal tract immune system with a pharmaceutically acceptable carrier;

[4] a method for assessing whether a test microorganism activates the intestinal tract immune system, comprising the steps of:

(a) preparing an extract from a test microorganism;

(b) contacting the extract with a cell forced to express an intestinal tract tissue-expressed Toll-like receptor; and

(c) measuring activity of the Toll-like receptor using signal transduction in the cell as an indicator,

wherein the test microorganism is judged to be activating the intestinal tract immune system if the activity of the Toll-like receptor is increased as compared to activity of the Toll-like receptor in a cell not contacted with the extract;

[5] a method of screening for a microorganism that activates the intestinal tract immune system, comprising the steps of:

(a) assessing whether a plurality of test microorganisms activate the intestinal tract immune system by the assessment method of [4]; and

(b) selecting from the plurality of test microorganisms those assessed to activate the intestinal tract immune system;

[6] a method for producing a food composition that activates the intestinal tract immune system, comprising the steps of [5], and a further step of mixing the microorganism assessed to activate the intestinal tract immune system with a dietarily acceptable carrier;

[7] the method of [6], wherein the microorganism is a lactic acid bacterium and the food composition is a dairy product;

[8] the method of any one of [4] to [6], wherein the microorganism is a lactic acid

bacterium;

[9] the method of [8], wherein the bacterium is a lactic acid bacterium;

[10] a method for constructing a model intestinal immunocompetent cell, comprising the step of introducing into a cell an expression vector comprising a DNA encoding an intestinal tract tissue-expressed Toll-like receptor;

[11] use of a cell forced to express an intestinal tract tissue-expressed Toll-like receptor as a model intestinal immunocompetent cell;

[12] the method of any one of [1] to [11], wherein the intestinal tract tissue is intestinal lymphoid tissue;

[13] the method of [12], wherein the intestinal lymphoid tissue is Peyer's patch or intestinal lymph node;

[14] the method of any one of [1] to [13], wherein the Toll-like receptor is derived from swine;

[15] the method of any one of [1] to [13], wherein the Toll-like receptor is Toll-like receptor 9;

[16] a cell forced to express an intestinal tract tissue-expressed Toll-like receptor for use in the method of any one of [1] to [9];

[17] a model intestinal immunocompetent cell constructed by introducing into a cell an expression vector comprising a DNA encoding an intestinal tract tissue-expressed Toll-like receptor;

[18] the cell of [16] or [17], wherein the intestinal tract tissue is intestinal lymphoid tissue;

[19] the cell of [18], wherein the intestinal lymphoid tissue is Peyer's patch or intestinal lymph node;

[20] the cell of any one of [16] to [19], wherein the Toll-like receptor is derived from swine; and

[21] the method of any one of [16] to [19], wherein the Toll-like receptor is Toll-like receptor 9.

Toll-like receptor 9 (TLR9), which is involved in the activation of immune response, has been known to be strongly expressed in the spleen (Zarember KA. and Godowski PJ. Journal of Immunology. 168 (2002) 554-561). In contrast, the present inventors discovered for the first time that TLR9 is strongly expressed in intestinal lymphoid tissues, in particular, the Peyer's patches and mesenteric lymph nodes.

The intestinal tract, a boundary tissue between the interior and the exterior of a human body, is constantly exposed to external stimuli (e.g., microorganisms, such as bacteria and viruses, drugs, food additives, residual pesticides in food, and environmental pollutants). Therefore, the intestinal tract serves not only to absorb orally ingested nutrients, but also as a

first biological defense mechanism (first defense line) in the receipt, transportation, response and elimination of foreign substances (Mantis NJ. *et al.*, J. Immunol. 169 (2002) 1844-1851). Other defense mechanisms including lymphoid tissues and gut-associated lymphoid tissues (GALT) are present. GALT is made up of diffusive compositions and aggregative compositions. The diffusive compositions include intestinal intraepithelial lymphocytes and lymphocytes of the lamina propria mucosae, and the aggregative compositions include the Peyer's patches, lymphoid follicles and mesenteric lymph nodes (Spahn TW. *et al.*, Eur. J. Immunol. 32 (2002): 1109-1113). The Peyer's patches are covered with follicle-associated epithelium (FAE) and form dorm-like elevations in villus-free areas. The patches include a follicular area where germinal center B-cells are present and a parafollicular area where helper T-cells are present (Owen RL. Sem. Immunol. 11 (1999) 157-163). Membranous epithelial cells (M-cells), which are specialized epithelial cells that serve as the first line of defense in the local immune mechanism of intestinal tract, are dispersed in FAE. M-cells have a deep pocket that serves as a "tunnel" in which antigens are transported through the cytoplasm to the basolateral side, where they are presented to antigen-presenting cells, including lymphocytes, dendritic cells and macrophages. M-cells have also been found in the epithelial mucosa of trachea and reported to serve as an entry site for pathogens such as Bacillus tuberculosis (Teitelbaum R. *et al.*, Immunity. 10 (1999) 641-650). M-cells are also known to serve as an entry site for functional factors contained in food products, as well as for microorganisms and food antigens. Once taken up by M-cells, intestinal luminal antigens (especially macromolecules) are transported to the inside of Peyer's patches, where they come into contact with major histocompatibility complex (MHC) class II-positive antigen-presenting cells such as dendritic cells and macrophages (Kaneko K. *et al.*, J. Veterinary. Med. Sci. 61 (1999) 1175-1177; Gebert A. *et al.*, American J. Pathology. 154 (1999) 1573-1582; Jensen VB. *et al.*, Infection & Immunity. 66 (1988) 3758-3766; Penheiter KL. *et al.*, Mol. Microbiol. 24 (1997) 697-709; Debard N. *et al.*, Gastroenterology. 120 (2001) 1173-1182; Gebert A. *et al.*, Int. Rev. Cytology. 167 (1996) 91-159). Upon antigen stimulation, helper T-cells produce Fc receptors, antigen-binding factors (IBF), IL-2, IL-4, IL-5 and IL-6. T-cells and B-cells which are activated upon antigen presentation then start "homing": migrate via mesenteric lymph nodes into the thymus and are then transported via circulation into tissues under action, such as intestinal lamina propria mucosae, mammary gland, lacrymal gland, salivary gland and urogenital organs. B-cells then become plasma cells to produce IgA. The secretory IgA acts to eliminate viruses, bacteria, bacteriotoxins and allergens that enter the intestinal tract and other mucous tissues (Vaerman JP. *et al.*, Immunology. 54 (1985) 601-603; Machtinger S. and Moss R., J. Allergy. Clinical. Immunol. 77 (1986) 341-347; Mathewson JJ. *et al*, J. Infectious Diseases. 169 (1994) 614-617). Mesenteric lymph nodes develop beneath the Peyer's patches, where more lymphocytes, dendritic cells and macrophages are present across the

Peyer's patches. The Peyer's patches and mesenteric lymph nodes thus play a central role in the intestinal tract immune system (immune system in the intestinal tract as above).

Meanwhile, the relation between intestinal mucosal epithelial cells and the uptake of FITC-labeled lipopolysaccharide (LPS), which is a known ligand of TLR4 and has been obtained from simian intestinal epithelium were analyzed using a TLR4 antibody and an IRAK antibody. The results indicated that LPS was taken up by the intestinal epithelial cells expressing TLR4 and IRAK and was transported to lamina propria mucosae (Imaeda H. *et al.*, Histochemical Cell Biology. 118 (2002) 381-388). This observation suggests intestinal tract tissue-expressed TLRs, such as TLR9, are involved in the intestinal tract immune system.

In view of the foregoing knowledge, the present invention provides methods for assessing whether a test sample activates the intestinal tract immune system or not. In this method, the test sample is first contacted with cells (TLR transfectant) that are forced to express an intestinal tract tissue-expressed TLR. In this step, the test sample is brought into contact with TLR on the surface of the transfectant. Activity of the TLR is then measured using signal transduction in the TLR transfectant as an indicator. In this assessment method, the test sample is judged to be activating the intestinal tract immune system if the TLR activity is increased as compared to that in a transfectant that has not been contacted with the test sample.

Examples of the test sample in the present invention include, but are not limited to, single compounds such as DNA, DNA fragments, natural compounds, organic compounds, inorganic compounds, proteins and peptides, and compound libraries, expression products of gene libraries, supernatants of nonmammalian cell cultures, extracts of nonmammalian cells, products of microbial fermentation, supernatants of microbial cultures, extracts of microorganisms, extracts of marine organisms and plant extracts. While the DNA fragments may be of any origin, those with a CpG motif, AT motif or CpG-like motif are preferred. Examples of microbial extracts include cell walls, cell membranes, DNA, RNA and flagella. Examples of microorganism include bacteria and yeast. Bacteria include pathogenic bacteria and lactobacillus. When necessary, the test sample may be labeled, for example, with a radiolabel or a fluorescent label.

The "intestinal tract" in the present invention includes, but is not limited to, duodenum, jejunum and ileum. While the intestinal tract tissue in the present invention may be any intestinal tract tissue, it is preferably an intestinal lymphoid tissue, more preferably a Peyer's patch or intestinal lymph node, and even more preferably a Peyer's patch or intestinal lymph node obtained from ileum.

In the present invention, the "intestinal tract tissue-expressed TLR" encompasses all types of TLRs, including, for example, TLR1 to TLR10. Ten different types of TLRs have been identified and reported up to now, each in the TLR family recognizing a different molecule, i.e.,

bacterial modulin. Bacterial modulins are defined as pathogen-associated molecular patterns (PAMPs) that show ability to induce cytokines in hosts and control their immune responses. TLRs are known to contain leucine-rich repeats (LRRs) on their extracellular domain and an intracellular TIR domain homologous to interleukin 1 receptor. Organisms from which the intestinal tract tissue-expressed TLR is derived include pigs, humans, mice, cats, vertebrates and invertebrates, and general organisms.

Examples of TLR9 in the present invention are proteins comprising the amino acid sequence shown in SEQ ID NO: 2, 4, 6 or 8. DNA coding for the proteins comprising the amino acid sequence shown in SEQ ID NO: 2, 4, 6 or 8 can be prepared by preparing RNA from thymus, lung, spleen, duodenum, Peyer's patch, mesenteric lymph node or such, synthesizing cDNA using reverse transcriptase, performing PCR to amplify the cDNA that codes for an above protein using oligo DNAs that have been synthesized based on SEQ ID NO: 1, 3, 5 or 7 as primers.

The "TLR9" in the present invention also includes proteins functionally equivalent to proteins comprising the amino acid sequence shown in SEQ ID NO: 2, 4, 6 or 8. Such proteins include mutants, alleles, variants and homologues of proteins comprising the amino acid sequence shown in SEQ ID NO: 2, 4, 6 or 8. As used herein, the term "functionally equivalent" means that a protein of interest has biological functions (biological roles) or biochemical functions (biochemical activities) equivalent to those of proteins comprising the amino acid sequence shown in SEQ ID NO: 2, 4, 6 or 8.

On recognizing microbial components, TLRs activate intracellular signal transduction pathways and facilitate translocation of IL-1 receptor-associated kinase (IRAK), TNF receptor-associated factor 6 (TRAF6) and transcription factor NF-κB to the nucleus, via a common adaptor molecule, MyD88. This ultimately induces production of various inflammatory cytokines, such as tumor necrosis factor α (TNF-α), interleukin (IL)-6, IL-12, IL-18 and IFN-γ, and expression of cell surface co-stimulatory molecules (Kaisho T. and Akira S. Trends in Immunology. 22 (2001) 78-83). Since TLRs primarily recognize pathogen carbohydrates, lipids and nucleic acids, they serve to compensate for the acquired immunity, which recognizes proteins.

The biological functions and biochemical functions of proteins comprising the amino acid sequence shown in SEQ ID NO: 2, 4, 6 or 8 include ability to recognize microbial components, ability to activate intracellular signal transduction pathways, ability to induce expression of inflammatory cytokines, and ability to induce expression of cell surface co-stimulatory molecules.

Methods for preparing DNAs encoding a protein functionally equivalent to a certain protein include methods using hybridization techniques (Sambrook, J *et al.*, "Molecular Cloning"

2nd ed., 9.47-9.58, Cold Spring Harbor Lab. Press, 1989). Specifically, the nucleotide sequence shown in SEQ ID NO: 1, 3, 5 or 7, or a fragment thereof, can be used to isolate DNAs encoding proteins that are functionally equivalent to a protein comprising the amino acid sequence shown in SEQ ID NO: 2, 4, 6 or 8.

One skilled in the art may appropriately select the hybridization conditions required for isolating DNAs encoding proteins functionally equivalent to a protein comprising the amino acid sequence shown in SEQ ID NO: 2, 4, 6 or 8, and hybridization conditions such as low-stringency conditions may be used. For low-stringency conditions, the after-hybridization wash is, for example, 42°C, 5 x SSC and 0.1% SDS, and more preferably 50°C, 5 x SSC and 0.1% SDS. More preferred hybridization conditions are high-stringency conditions, for example, 65°C, 0.1 x SSC and 0.1% SDS. Under these conditions, DNAs with higher homology can be efficiently obtained at higher temperatures. It should be noted that a number of parameters affecting the stringency of hybridization, such as temperature and salt concentration, are considered. Those skilled in the art can choose these parameters appropriately to achieve similar stringency.

Alternatively, using primers synthesized based on the sequence information shown in SEQ ID NO: 1, 3, 5 or 7, DNAs encoding proteins functionally equivalent to a protein comprising the amino acid sequence shown in SEQ ID NO: 2, 4, 6 or 8 may be isolated by gene amplification techniques, such as polymerase chain reaction (PCR).

Proteins that are encoded by DNAs isolated using hybridization or gene amplification techniques, and which are functionally equivalent to a protein comprising the amino acid sequence shown in SEQ ID NO: 2, 4, 6 or 8, generally have high homologies to a protein comprising the amino acid sequence shown in SEQ ID NO: 2, 4, 6 or 8 at the amino acid level. The term "high homology" used herein means at least 60% homology, preferably 70% or higher homology, more preferably 80% or higher homology, more preferably 90% or higher homology, still more preferably 95% or higher homology, and most preferably 98% or higher homology at the amino acid level.

The similarity of amino acid sequences or nucleotide sequences can be determined using the BLAST algorithm by Karlin and Altschul (Proc. Natl. Acad. Sci. USA 90:5873-5877, 1993). Programs such as BLASTN and BLASTX have been developed based on this algorithm (Altschul *et al.*, J. Mol. Biol. 215:403-410, 1990). When nucleotide sequences are analyzed by the BLAST-based BLASTN, parameters are set to, for example, score = 100 and word-length = 12. When amino acid sequences are analyzed by the BLAST-based BLASTX, parameters are set to, for example, score = 50 and word-length = 3. When BLAST is used in conjunction with the Gapped BLAST program, default parameters of each program are used. Specific procedures of these analytical techniques are known (http://www.ncbi.nlm.nih.gov.).

The present invention encompasses proteins comprising an amino acid sequence with

one or more amino acid mutations in the amino acid sequence shown in SEQ ID NO: 2, 4, 6 or 8. Such amino acid mutations can occur naturally. The number of amino acid mutations is typically 30 amino acids or less, preferably 15 amino acids or less, more preferably 5 amino acids or less, and still more preferably 2 amino acids or less.

The methods of the present invention use the above-described TLR transfectant. The TLR transfectant can be obtained by introducing into a cell an expression vector that comprises a DNA encoding an intestinal tract tissue-expressed TLR. Examples of such an expression vector include mammalian expression vectors (e.g., pcDNA3 (Invitrogen), pEGF-BOS (Nucleic Acids. Res. 1990, 18(17), p5322), pEF and pCDM8), insect expression vectors (e.g., "Bac-to-BAC baculovirus expression system" (Invitrogen) and pBacPAK8), animal virus expression vectors (e.g., pHSV, pMV and pAdexLcw) and retroviral expression vectors (e.g., pZIPneo).

For expression in animal cells such as CHO cells, COS cells and NIH3T3 cells, the expression vector must carry a suitable promoter for cellular expression. Examples of such promoters include SV40 promoter (Mulligan *et al.*, Nature (1979) 277, 108), MMLV-LTR promoter, EF1α promoter (Mizushima *et al.*, Nucleic Acids Res. (1990) 18, 5322) and CMV promoter. More preferably, the expression vector may have a gene for selection of transformed cells (for example, a drug resistance gene that allows selection by a drug such as neomycin or G418).

To ensure stable expression of a gene and amplify copy number of the gene in cells, the gene may be amplified by introducing a vector (e.g., pCHOI) having a complementary dihydrofolate reductase gene (dhfr gene) introduced into CHO cells, whose nucleic acid synthesis pathways are defective, and using methotrexate (MTX). Methods for transiently expressing a gene include those that transform COS cells, which carry an SV40 T antigen-expressing gene on their chromosome, with a vector having an SV40 replication origin (e.g., pcD). The replication origin may be obtained from polyomavirus, adenovirus bovine papillomavirus (BPV) or such. The expression vector may further comprise a selection marker, such as aminoglycoside transferase (APH) gene, thymidine kinase (TK) gene or dhfr gene, to amplify the gene copy number in host cell system.

The host cells include, but are not limited to, mammalian cells and insect cells. Known examples of mammalian cells include HEK293T cells, CHO (J. Exp. Med (1995) 108, 945), COS, NIH3T3, myeloma, BHK (baby hamster kidney), HeLa and Vero. Known examples of insect cells include Sf9, Sf21 and Tn5.

CHO cells, in particular, dhfr-CHO having a defective dhfr gene (Proc. Natl. Acad. Sci. USA (1980) 77, 4216-4220) and CHO K-1 (Proc. Natl. Acad. Sci. USA (1968) 60, 1275), are preferably used. CHO cells are particularly preferred when the objective is to have abundant expression of a gene in animal cells. In addition, immortalized cell lines are preferred host

cells.

The vector can be introduced into host cells using methods known by one skilled in the art, including, for example, calcium phosphate, DEAE dextran, cationic ribosome DOTAP (Roche Diagnostics), electroporation, and lipofection.

In the present invention, the TLR activity is measured by using signal transduction in the above-described transfectant as an indicator. For example, the TLR activity can be measured by using expression levels of cytokines (e.g., IL-6, IL-12, IFN-γ and TNF-α), activation of molecules involved in signal transduction pathways (e.g., NF-κB, JNK and IRAK), or such as an indicator. The expression of a cytokine can be detected at the mRNA or protein level. For example, the expression of a cytokine at the protein level can be measured by using existing kits for human, including human IL-6, IL-12, TNF-α and IFN-γ ELISA kits (TRB, INC.). Increase in the activities of molecules in signal transduction pathways can be detected by luciferase assay. For example, a swine TLR transfectant is transfected with a plasmid vector (pGLM-ENH) comprising NF-κB and a luciferase gene, and stimulated with a ligand (e.g., DNA) 18 hours later. The cells are lysed 24 hours after stimulation, and then the cell lysate is collected and stored at -80°Ç until use. Tthe luciferase activity is determined by adding a luciferin-containing reaction solution to the cell lysate, and measuring the change in luminescence over an eight-second period starting at 2 seconds after the addition. The same procedure is repeated three times for each sample and averages are taken. Higher luminescence intensity indicates stronger NF-κB activity.

The present invention also provides methods of screening for samples that activate the intestinal tract immune system. In the screening methods of the present invention, a plurality of test samples are assessed for the ability to activate the intestinal tract immune system, using the above-described assessment methods. Samples that are assessed as activating the intestinal tract immune system are selected.

Further, samples that bind to an intestinal tract tissue-expressed TLR may be screened in advance and used as test samples. The TLR used to screen for samples that bind to an intestinal tract tissue-expressed TLR may be a recombinant protein or a naturally occurring protein. The TLR used in screening may be a partial peptide. To screen for samples that bind to an intestinal tract tissue-expressed TLR, a plurality of test samples are first contacted with an intestinal tract tissue-expressed TLR. Binding of the TLR with the test samples is detected. Test samples that bind to the TLR are then selected. The binding of the TLR with the test samples can be detected by methods known to one skilled in the art.

The samples obtained in the above-described assessment methods or screening methods can be used as samples having an immunostimulatory function in the treatment or prevention of diseases, for example, allergies, cancers, and infections.

The present invention further provides methods for producing pharmaceutical compositions that activate the intestinal tract immune system. The pharmaceutical compositions of the present invention can be used as pharmaceutical compositions having an immunostimulatory function in the treatment or prevention of diseases, for example, allergies, cancers, and infections. The pharmaceutical compositions of the present invention are preferably uses as vaccines.

In methods for producing the pharmaceutical compositions of the present invention, a sample that has been assessed as activating the intestinal tract immune system by the above-described screening methods is mixed with a pharmaceutically acceptable carrier. One example of such a pharmaceutically acceptable carrier is adjuvant (antibody production potentiator). Other examples of pharmaceutically acceptable carriers include surfactants, excipients, coloring agents, flavoring agents, preservatives, stabilizing agents, buffering agents, suspending agents, isotonizing agents, binding agents, disintegrating agents, lubricants, fluidity accelerators, and corrigents. Other commonly used carriers may also be used suitably. Specific examples include light silicic acid anhydride, lactose, crystalline cellulose, mannitol, starch, carmellose calcium, carmellose sodium, hydroxypropylcellulose, hydroxypropylmethylcellulose, polyvinylacetal diethylaminoacetate, polyvinylpyrrolidone, gelatin, middle-chain fatty acid triglycerides, polyoxyethylene hydrogenated castor oil 60, sucrose, carboxymethylcellulose, corn starch and inorganic salts. The thus produced pharmaceutical compositions which activate the intestinal tract immune system can be used as oral agents or injections.

The present invention further provides methods for assessing whether a test microorganism activates the intestinal tract immune system. Examples of microorganisms in the present invention include, but are not limited to, bacteria and yeast. The bacteria include, but are not limited to, LAB (e.g., LAB that is indigenous to the intestines and dairy LAB).

In the assessment methods, extracts are prepared from a test microorganism. Examples of such microorganism extracts include cell walls, cell membranes, DNAs, RNAs and flagella. Preferred DNAs are fragments containing a CpG motif, AT motif or CpG-like motif. Such extracts can be prepared from the microorganisms by methods known by one skilled in the art.

An example of the method for preparing fragments containing a CpG motif, AT motif or CpG-like motif from dairy LAB is shown below, but methods of the present invention are not limited thereto.

Lactobacilli and Streptococci are subcultured (37°C, 24 hours) three times in Lactobacilli MRS broth (Difco Laboratories, Detroit, MI, USA) and in Elliker broth, respectively. Subsequently, the bacteria are inoculated at 1% in a 50 ml medium and are cultured at 37°C for 16 hours. The cells are then collected by centrifugation (3,000 xg, 4°C, 20 min), washed twice

by centrifugation (4,000 xg, 4°C, 20 min) in TE buffer (10 mM Tris-Cl, 1 mM EDTA pH 7.5), and then resuspended in 5.0 mL of TE buffer. To this suspension, 2.5 ml of lysozyme (30 mg/ml, Seikagaku Co. Ltd., Tokyo) and 20 μl of N-acetylmuramidase SG (250 μg/ml, Seikagaku Co. Ltd., Tokyo) are added and the reaction is carried out at 37°C for 10 to 30 minutes. To this suspension, 10 ml of 0.1 M Tris-1% SDS solution is then added and the solution was gently stirred. Subsequently, a proteinase K solution (20 mg/ml, 150 μl, TaKaRa, Kyoto) is then added and stirred, and the reaction is carried out at 37°C overnight. To this mixture, 5 M NaCl solution (2.5 ml) is added and the mixture is transferred to a sterilized beaker. 100% ethanol (50 ml) is then added to precipitate the nucleic acid. The resulting precipitate is picked up using a sterilized glass rod, washed with 70% ethanol, and dissolved in TE buffer (10 ml). The solution is allowed to stand at 4°C overnight to completely dissolve the precipitate. Following addition of RNase A (10 mg/ml, 100 μl, SIGMA) and subsequent incubation at 37°C for 60 min, 1/10 volume of 5 M NaCl (1 ml) and an equal volume of 100% ethanol are added to precipitate the nucleic acid. The resulting precipitate is wound onto a sterilized glass rod, washed with 70% ethanol, and dissolved in a TE buffer (20 ml). The thus purified DNA is then stored at 4°C until use. The chromosomal DNA is digested with restriction enzyme Sau 3AI and the digest is subjected to 3% agarose gel electrophoresis. The DNA collected from the agarose gel is ligated into a plasmid vector. Sequence of the cloned DNA is then determined on a sequencer to prepare fragments having a CpG motif, AT motif or CpG-like motif.

In the assessment methods, the TLR transfectant is contacted with the extract, and activity of the intestinal tract tissue-expressed TLR is measured by using signal transduction in the transfectant as an indicator. The test microorganism is judged as activating the intestinal tract immune system if the TLR activity is increased when compared to the activity in cells that are not contacted with the extract.

The present invention further provides methods of screening for microorganisms that activate the intestinal tract immune system. In these screening methods, the above-described assessment methods are used to evaluate multiple test microorganisms for their ability to activate the intestinal tract immune system, and the microorganisms evaluated as activating the intestinal tract immune system are selected.

The microorganisms obtained in the assessment methods or screening methods can be used as microorganisms having immunostimulatory function in the treatment or prevention of diseases, for example, allergies, cancers, and infections.

The microorganisms which activate the intestinal tract immune system can be used to make food compositions that activate the intestinal tract immune system. The present invention also provides methods for producing food compositions that activate the intestinal tract immune system. In methods for producing food compositions of the present invention, a microorganism

that has been evaluated as activating the intestinal tract immune system by the above-described screening method is mixed with a dietarily acceptable carrier. Examples of a dietarily acceptable carrier include stabilizing agents, preservatives, coloring agents and flavoring agents.

Preferred embodiments of the food compositions of the present invention include food and beverage products containing LAB or yeast. Examples of such food and beverage products containing LAB or yeast include dairy products. Examples of the dairy products of the present invention include fermented milk, cheese, and fermented food products (lactobacillus-containing food products, and Kimchi, etc.). These products can be produced by methods known by one skilled in the art.

The food compositions thus produced can be used in the treatment or prevention of diseases, for example, allergies, cancers, and infections as food products having immunostimulatory function (for example, functional food products, health food products, and food for specified health uses).

The present inventors discovered for the first time the involvement of TLRs in intestinal tract immunity. TLRs have also been known to express in cells responsible for innate immunity (for example, macrophages and dendritic cells) (Gordon S. Cell. 111 (2002) 927-930, Akira S. *et al.*, Nature Immunology. 2 (2001) 675-680). Thus, the TLR transfectants of the present invention can be used in the above-described methods of the present invention as models of intestinal immunocompetent cells. In addition, the TLR transfectants of the present invention, along with the other components used in the methods of the present invention, can be used as kits for the methods of the present invention.

The present invention further provides TLR transfectants that can be used in the methods of the present invention, methods for using the TLR transfectants as models of intestinal immunocompetent cells, methods for producing model cells of the intestinal immunocompetent cells, and the model cells produced by such production methods.

Brief Description of the Drawings

Fig. 1 shows the nucleotide sequence of swine TLR9 cDNA. The signal peptide region is underlined.

Fig. 2 shows the nucleotide sequence of swine TLR9 cDNA (Continuation of Fig. 1).

Fig. 3 shows the nucleotide sequence of swine TLR9 cDNA (Continuation of Fig. 2).

Fig. 4 shows the nucleotide sequence of swine TLR9 cDNA (Continuation of Fig. 3). The transmembrane domain is underlined.

Fig. 5 shows the results of the domain analysis of swine TLR9 by SMART.

Fig. 6 shows an alignment of TLR9 amino acid sequences.

Fig. 7 shows an alignment of TLR9 amino acid sequences (Continuation of Fig. 6).

Fig. 8 shows an alignment of TLR9 amino acid sequences (Continuation of Fig. 7).

Fig. 9 shows an alignment of TLR9 amino acid sequences (Continuation of Fig. 9).

Fig. 10 shows photographs of the expression analysis results of swine TLR9 in transfectant using RT-PCR.

Fig. 11 shows the analysis results of swine TLR9 expression and CpG DNA uptake in transfectant. a: analysis using anti-FLAG antibody. b: analysis using anti-sTLR9 antibody. c: analysis of CpG DNA uptake. Arrow (1) indicates the uptake of CpG1826 and CpG2006 by control cells. Arrow (2) indicates the uptake of CpG1826 in the transfectant. Arrow (3) indicates the uptake of CpG2006 by the transfectant.

Fig. 12 shows photographs of the analysis results of the transfectants by confocal laser microscopy. a, b, c: control cells; d, e, f: swine TLR9 transfectants. a, d: analysis using anti-FLAG antibody. b, e: analysis using anti-sTLR9 antibody. c, f: analysis of CpG DNA uptake.

Fig. 13 shows results of the expression analysis of sTLR9 in different tissues using real-time quantitative PCR.

Best Mode for Carrying Out the Invention

Herein below, the present invention will be specifically described using Examples; however, it is not to be construed as being limited thereto.

1) Swine tissue

Swine tissue was purchased from Funakoshi Co. Ltd.

2) Cloning and nucleotide sequencing of swine TLR9 gene

Primers were prepared based on the highly conserved region in the gene sequences of human TLR9 and mouse TLR9 published by DDBJ/EMBL/GenBank (Accession Numbers AB045180 and AF348140, respectively). The primers were used in RT-PCR to obtain swine TLR9 fragments from total RNA of the Peyer's patch of swine intestinal tract. The gene fragments were subcloned by ligating into pGEM-T-Easy vector and transfecting E.coli JM 109 competent cells with the vector. The DNA sequence was determined using a DNA sequencer Model 4000L (Li-Cor, Lincoln, NE, USA). The nucleotide sequence and amino acid sequence were analyzed using GENETYX-SV/RC Ver. 11.0.3.1. The rest of the swine TLR9 gene sequence was obtained by RACE using primers designed from the swine TLR9 gene fragments. The entire TLR9 gene was amplified by PCR and cloned.

3) Preparation of swine TLR9-specific polyclonal antibody

Results in antigenic determinant (epitope) analysis using GENETYX-SV/RC Ver.

11.0.3.1 and secondary structure analysis of protein showed that the region from amino acids 268 to 284 of swine TLR9 have high antigenicity. We asked Sawady Technologies Co Ltd. for the synthesis of the peptide of that region and preparation of a polyclonal antibody that recognizes the synthetic peptide as antigen.

4) Construction of swine TLR9 transfectant

HEK293T (Human Embryonic Kidney) cells, a widely used human cell line for gene introduction, were chosen as the host for introduction of the swine TLR9 gene. Human TLR9-specific primers were used to confirm that HEK293T cells did not express the TLR9 gene. The swine TLR9 gene, excluding the signal peptide, was ligated to pCXN2.1-FLAG gene expression vector (H. Niwa *et al.*, Gene, 108 (1991) 193-199) (courtesy of Dr. Jun-ichi Miyazaki at Osaka University, Graduate School of Medicine, Faculty of Medicine), and HEK293T cells were transfected with the vector by lipofection. Cells that express swine TLR9 were selected on EPICS cell sorter system (BECKMAN COULTER) using antibiotic G418 neomycin (SIGMA).

5) RT-PCR analysis of swine TLR9 expression in the transfectant

Using TRIzol (Invitrogen), total RNA was extracted from the transfectant. Gene expression of TLR9 mRNA was analyzed by RT-PCR using swine TLR9-specific primers (designed by the present invention), human TLR9-specific primers, and human GAPDH primers (positive control) (K. A. Zarember, P. J. Godowski, J. Immunology, 168 (2002) 554-561).

6) Flow cytometry and confocal laser microscopy analyses of swine TLR9 expression in the transfectant

The cells were first treated with an anti-FLAG mouse IgG monoclonal antibody (SIGMA) as a primary antibody at 4°C for 1 hour and then stained with a PerCP-labeled anti-mouse IgG antibody as a secondary antibody at 4°C for 30 min. The cell nuclei were stained with propidium iodide at 4°C for 10 min. Analysis was carried out using FACSCalibur™ (JAPAN BECTON DICKINSON). Immunostaining with the swine TLR9 polyclonal antibody is carried out by first treating the cells with the primary antibody mentioned above and subsequently staining with an Alexa 488-labeled anti-rabbit IgG antibody as a secondary antibody at 4°C for 30 min. The cell nuclei were stained and the cells were analyzed. In addition, the cells were seeded onto type-I collagen-coated disks (IWAKI), treated with a biotin-labeled anti-FLAG mouse IgG antibody (SIGMA) as a primary antibody at 4°C for 1 hour, and then stained with streptavidin-PE-Cy5 antibody as a secondary antibody at 4°C for 30 min. The cell nuclei were stained with propidium iodide at 4°C for 10 min. Analysis was carried out

using a confocal laser microscope (BIO-RAD).

7) Analysis of CpG DNA uptake using the transfectant

CpG DNAs used were: CpG2006 derived from E.coli genomic DNA reported to strongly stimulate human immune cells (SEQ ID NO: 9, 5'-TCGTCGTTTTGTCGTTTTGTCGTT-3'); and CpG1826 reported to strongly stimulate mouse immune cells (SEQ ID NO: 10, 5'-TCCATGACGTTCCTGACGTT-3') (S. Pichyangkul *et al.*, J. Immunological Methods, 247 (2001) 83-94). The cells were incubated with 1M CpG DNA at 37°C for 1 hour and analyzed using FACSCalibur™ and confocal laser microscope.

8) Real-time PCR analysis of TLR9 expression in different swine tissues.

Total RNA was extracted form different swine tissues (i.e., heart, thymus, lung, spleen, liver, kidney, skeletal muscle, duodenum, jejunum, ileum, ileal Peyer's Patch, and ileal mesenteric lymph node). Using oligo-$d(T)_{18}$ primers, cDNA was synthesized from 1 μg of total RNA and purified. Real-time quantitative PCR was performed on a LightCycler (Roche) using the swine TLR9-specific primers and the purified cDNA. Light Cycler-Fast Start DNA Master SYBR Green (Roche) was used as the reaction kit. The amount of swine TLR9 mRNA was calculated from the ratio of the amount of swine TLR9 gene to the amount of the housekeeping β-actin gene determined from the calibration curve. By setting the expression level of TLR9 in spleen to be 1.000, the amounts of swine TLR9 mRNA in different tissues were compared.

The nucleotide sequences of the primers used in the present invention are summarized in Table 1.

[Table 1]

sTLR9 gene cloning primers	Forward primers	SEQ ID NO:	Reverse primers	SEQ ID NO:
sTLR9(2775-3145)	AGACTGGTTACCTGGCAAGA	11	GCTATTCDGCDGTDGGAC	12
sTLR9(2301-2775)	CAACCTGAAAGTCCTAGACG	13	GGCAGAAGTTCCGGTTATAG	14
sTLR9(1708-2347)	AGCTACAACAGCCAGCCCTT	15	AGGCGCAGTGCAGAGGGTT	16
sTLR9(1057-1727)	CTGCGCAAGCTCAACCTGT	17	AAGGGCTGGCTGTTGTAGCT	18
sTLR9(140-1089)	CTGCCTTCCTACCCTGTGA	19	GTGGTAATTGAAGGACAGGTT	20
sTLR9(5'RACE, cDNA synthesis)			GCAGTTCCACTTGAGGTT GA	21
sTLR9(5'RACE, 1st nested PCR)			ACGAAGTCAGAGTCGTGCAA	22
sTLR9(5'RACE, 2nd nested PCR)			AGGAAGAGCCAGTTGCAGTT	23
Primers for analysis of mRNA expression	Forward primers	SEQ ID NO:	Reverse primers	SEQ ID NO:
swine TLR9	CTGAAAGTCCTAGACGTGAG	24	TCTTGCCAGGTAACCAGTCT	25
human TLR9	GGACCTCTGGTACTGCTTCCA	26	AAGCTCGTTGTACACCCAGTCT	27
human GAPDH	GAAGGTGAAGGTCGGAGTC	28	GAAGATGGTGATGGGATTTC	29
sTLR9 real-time RT-PCR	GTGGAACTGTTTTGGCATC	30	CACAGCACTCTGAGCTTTGT	31
sβ-actin real-time RT-PCR	TGGCATTGTCATGGACTCTG	32	AGGGGCGATGATCTTGATCT	33

[Example 1] Determination of swine TLR9 gene sequence and its homology to TLR9s of other species

In the present invention, the swine TLR9 cDNA sequence determined has 3145 bases (with 54 bases of untranscriptional region at the 5'-end) containing 3090 bases of structural gene (ORF). The ORF encodes 1029 amino acid residues and has a molecular weight of 115.8 kDa (Figs. 1 to 4). TLR9s of various species were aligned in Figs. 6 to 9. The amino acid sequence of swine TLR9 had 82.0%, 74.9% and 86.6% homology to human, mouse and cat TLR9s, respectively (Table 2).

[Table 2]

TLR9	nucleotide sequence[a] homology (%)	amino acid sequence[a] homology (%)
human	84.9	82.0
mouse	78.2	74.9
cat	86.6	86.6

[a] The sequence information of human, mouse and cat TLR9s can be obtained at DDBJ by accession NOs AB0452180, AF348140 and AY137581, respectively.

[Example 2] Expression analysis of swine TLR9 transfectant

RT-PCT analysis of the expressions of swine TLR9 mRNA and human TLR9 mRNA, using total RNAs obtained from a control cell and the sTLR9 transfectant as templates, revealed strong expression of the swine TLR9 mRNA in the transfectant. Human TLR9 mRNA expression was detected in neither (Fig. 10).

When an anti-FLAG antibody was used as the primary antibody, the expression analysis by flow cytometry showed a large shift towards the positive side as compared to the control cell(Fig. 11-a). A shift towards the positive side was also observed for the swine TLR9 antibody (Fig. 11-b). Similarly, the expression was detected by laser microscopy (Fig. 12-a, c, b and d).

[Example 3] Analysis of CpG DNA uptake

While no significant difference was observed by confocal laser microscopy in the uptake of different CpG DNAs, the flow cytometry of CpG DNA uptake revealed that the swine TLR9 transfectant incorporated relatively larger amounts of human CpG2006 than mouse CpG1826 (Figs. 11-c, 12-e and f).

[Example 4] Analysis of swine TLR9 mRNA expression in different tissues

The real-time PCR analysis of swine TLR9 mRNA expression in different tissues showed that swine TLR9 was strongly expressed in the intestinal lymphoid tissue, especially in the Peyer's patch and mesenteric lymph node (Fig. 13).

Examples of the present invention have demonstrated that swine TLR9 has a higher homology to human or cat TLR9 than to mouse TLR9. Previous reports on the expression analysis by real-time PCR indicated that strong expression of TLR9 was observed in spleen. To the contrary, our results prove that the mRNA expression level is about three or more times higher in the Peyer's patches and mesenteric lymph nodes than in spleen. This finding is very interesting in view of the fact that the intestinal mucosal system, which is most likely to be exposed to orally ingested pathogenic bacteria, Peyer's patches and mesenteric lymph nodes play a crucial role in the intestinal immunity. In these Examples, the present inventors have successfully constructed a swine TLR9 transfectant and by using this transfectant in the analysis of CpG DNA uptake, demonstrated that swine TLR9 is more reactive to human CpG DNA than to mouse CpG DNA. These transfectants should serve as a driving force for future analyses of the recognizing ability for the DNA from functional LAB. Through the use of a swine experimental animal as a human model, these transfectants also allow detailed investigation of fundamental studies on the development of functional food products at the molecular level.

Bacterial DNA recognized by TLR9 is known to stimulate macrophages and dendritic cells and promote the production of cytokines and such (M. Bauer *et al.*, J. Immunol. 166 (2001) 5000-5007). The results of these examples are certain to lead to the development of vaccines that take advantage of stimulation by bacterial DNA and recognition by TLR9, as well as of signal transduction systems (R. L. Modlin, Nature 408 (2000) 659-660). Such vaccines can elicit various immune responses, from weak responses that are just enough to prevent infections to highly effective responses that can kill bacteria, and are thus expected to find wide applications in the treatment of tuberculosis and such, as well as cancers, allergies, and such. LAB has recently attracted much attention as an antigen carrier that reaches the intestinal tract alive. In addition to that, the DNA of LAB is shown to have immunostimulatory ability by the present invention and this has substantially raised the expectation of LAB. The present invention has demonstrated strong expression of TLR9 in the Peyer's patches and mesenteric lymph nodes - a strong indication of the important role of TLR9 in the intestinal tract immune system and an implication that the DNAs of intestinal LAB and dietary dairy LAB can stimulate TLR9 and thus activate the immune system. Thus, understanding the TLR9-mediated intestinal tract immune system by LAB DNA is a task that must be accomplished for developing DNA vaccines using intestinal indigenous LAB and dairy LAB.

The strong expression of TLR9 in the Peyer's patches and mesenteric lymph nodes may suggest significant development of an innate immune system in intestinal tract immunity. The intestinal tract immune system has recently received great attention in medical and immunological fields as a critical area of study. Nonetheless, intestinal tract immunity as a field of study is relatively new and little is known about its fundamental mechanisms. Thus, there is much to expect from understanding the mechanisms of the intestinal tract immune system.

The findings of the present invention provide clues to understanding the molecular mechanism of TLR9-mediated recognition in the signal transduction pathways of intestinal tract and serve as a driving force to promote the progress in understanding the innate immunity - a basic immune system.

Industrial Applicability

The present invention provides uses of TLR transfectants. The TLR transfectants can be used as model cells of intestinal immunocompetent cells. By using the TLR transfectants, samples and microorganisms that activate the intestinal tract immune system can be identified, and pharmaceutical compositions and food compositions that activate the intestinal tract immune system can be produced. The samples, microorganisms, pharmaceutical compositions and food compositions can be used in the treatment or prevention of diseases, for example, allergies, cancers, and infections.